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RESVERATROL RADIOMODIFIER EFFECT ON *DANIO RERIO* EMBRIOLARVAL ASSAY

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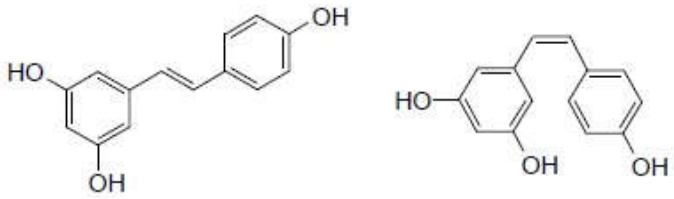
ABSTRACT

The ionizing radiation can cause fatal damages to cells by the direct interaction with DNA and RNA or a series of toxic reactions occasioning chemical and biological changes. There are compounds with radioprotective potential, like resveratrol. For use these compounds it is necessary to know their toxicity and interaction with the organism. Resveratrol is a substance found in peanuts, grapes and wine and its production occurs in plants as a response to physical, chemical and biological stress. Some studies have indicated that it has many health benefits. *Danio rerio* (zebrafish) is a vertebrate animal and has become the model of several studies related to human diseases, due to its genomes similarity of 70 %, rapid embryonic development and the transparency of the eggs, which make it possible to observe the effects during the test period. The aim of the present study was to verify the resveratrol radiomodifier effect on zebrafish during the embryolarval development by modified Fish Embryo Acute Toxicity (FET) based on OECD236 and the obtained lethal concentration of resveratrol (LC50) was 66.9 $mg.L^{-1}$. Before, to understand the effects of radiation, was carried out the gamma radiation lethal dose (LD50) assay and the LD50 was 25 Gy. With these results the project will continue later to finish the study of the radiomodifier effect of resveratrol in the presence of gamma radiation..

1. INTRODUCTION

Many vegetables contains phenolic compounds synthesized by plants in response to mechanic stress or fungal attack. Resveratrol is identified as the biggest active compound of stilbene phytoalexins and is expected to bring benefits of human health. The trans-resveratrol was first detected in 1976 in grapevines (Vitis viniferu) and it was found that the compound was synthesized by the leaf tissues in response to fungal infection (mainly *Botrytis cinereu*) or exposure to ultraviolet light.It is possible to find two isomers of the moleculethe trans(trans-3,5,4',5 trihidroxiestilbeno) and cis-resveratrol (cis-3,5,4'-trihidroxiestilbeno), presented in the Fig.1 [1].



Trans-**R** *Cis*-**R**

Figure 1: Isomers structure of resveratrol at both forms the trans (trans-3,5,4',5-trihidroxiestilbeno) and cis (cis-3,5,4'-trihidroxiestilbeno).

It is known that the isomer form of trans resveratrol has biological activity and it is common to find it in some species of peanuts and grapes. Some studies indicate many health benefits as vasodilatation, help in the metabolism of lipoproteins, inhibition of platelet aggregation and has therapeutic and preventive action on cancer. In addition to these effects, trans-resveratrol triggers a series of cellular and molecular effects [3].

Other important characteristic is the low toxicity that is of great interest to be used as a radioprotection of normal cells and in the prevention of cancer [4].

According to Moreno [5], when mammalian cell culture was exposed to gamma radiation they were more resistant in the presence of resveratrol.Magalhães et al [6] and Moreno [5] observed radiomodifying effect of resveratrol on carcinogenic cells when irradiated with ionizing radiation, dependent of resveratrol concentration.

The *in vivo* model *Danio rerio* known as zebrafish had been used in many studies for some reasons:easy to maintain in the laboratory under the right conditions, reproduceeasilywith a lot of eggs that are transparent, making possible to see and followthe rapid development of the embryos. The very important reason is that around 70% ofits genome is similar to the human and this make this organism as a model for many biomedical researches [7].The human and zebrafish genome is similar especially in key genes related to development, signal transduction, cell cycle progression and proliferation and cell differentiation.In both organisms, the effects of ionizing radiation and the phenotypical protection of radioprotective substances are very similar [8].

The radiation interaction with the organism can occur in two different ways, the direct where the incidence is in the DNA and RNA and can provoke fatal damages to the cells, when a apoptosis process arenot induced, the lesions can be incorrigible and a process of cellular neoplasm can begin, in this case the informationis passed through future generations [6]. Since radiation toxicity and the radiomodifying action of resveratrol on cells are already known in *in vitro* assays, but*in vivo* studies are required for the understanding the effects on the organisms.

2. MATERIALS AND METHODS

A preliminary test was carried out to obtainthe 50% Lethal Dose (LD_{50}) of gamma radiation in *Danio rerio* embryos and to subsequently determine the doses to be used in the definitive test.The procedure was an adaptation of the OECD 236 protocol [9].

The fertilized organisms were selected and collected in the laboratory of Toxinologia aplicada do Instituto Butantan.

20 embryos of 24hpf (hours post fertilization) conditioned in each 24well of cell culture plates with 2 mL of MS medium for 24 hours. The plates were irradiated in a source of Co-60(GammaCell 200) at 10, 20, 30, 40 and 50 Gy doses with dose rate of 0.773 $kGy.h^{-1}$.

After irradiation, the plates were conditioned in incubator with photoperiod of 12h light / 12h dark for more 5 days. Every 24 h, the indicators of lethality were observed and recorded: coagulation, absence of heart beat, absence of somite formation and no detachment of the tail of the yolk sac.

After this period, the LD_{50} was calculated in the statistical program Trimmed Spearman-Karber [10].

In parallel, *in vitro* cytotoxicity assay was performed to determine the cytotoxicity index (IC_{50}) of resveratrol following International StandardISO 10993-5 [11]. This assay was performed by the neutral red uptake method. Diluted solutions of resveratrol were placed in contact with NCTC clone 929 (CCIAL 020) mouse connective tissue cells from Instituto Adolfo Lutz, adhered to the 96 wells of the cell culture microplate. The microplates were supplied by the Núcleo de Culturas Celulares of the Instituto Adolfo Lutz. In the assay, in addition to cell control, a negative and a positive control were tested. Cell viability was verified by the incorporation of neutral red by the living and intact cells. The optical density reading of the final microplate solution was performed in a spectrophotometer, ELISA reader-SUNRISE, at 540nm, after cell lysis. The percentage of cell viability was calculated in relation to the control cells and projected on a graphic as a function of the concentration of resveratrol obtaining a curve that indicated the IC_{50}.

According to the cytotoxicty index (IC_{50}) of resveratrol it was determined the concentration to be used in the acute toxicity test with *Danio rerio* embryos based on OECD Protocol 236 [9]. Fresh fertilized eggs were selected and exposed to five concentrations of resveratrol 6.25;12.5;25;50 and 100 $mg.L^{-1}$during 96h in 24well polystyrene plates with one embryo per well containing 2 ml of solution andone plate per concentration.

Every 24 h, indicators of lethality: coagulation, absence of somite formation, absence of heart beats and tail detachment were observed and recorded. At the end of the exposure period the LC_{50} was calculated with number of dead organisms in the Trimmed Spearman-Karber statistical program [10].

3. RESULTS AND DISCUSSION

3.1 LD50 of gamma radiation

Figure 2. shows the results ofLD50 of gamma radiation in the 24hpf zebrafish embryos. The effect observed for lethality in this work was the absence of heart beat.

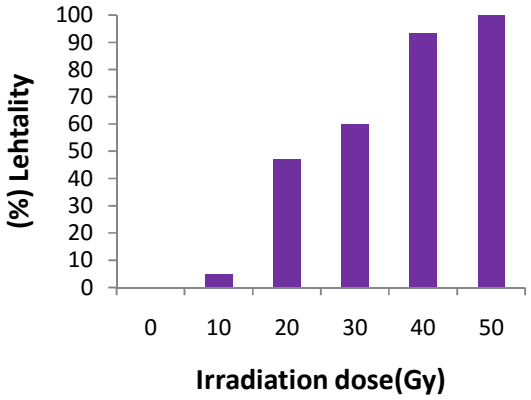


Figure 2: Results of LD50 of gamma radiation on zebrafish embryos assay.

The LD50 result obtained was 24.87Gy,similar to that of Daroczi, which the LD50 of gamma radiation in *Danio rerio*was 20 Gy and in the dose of 40 Gy caused the dead of 100% of the organism [12].

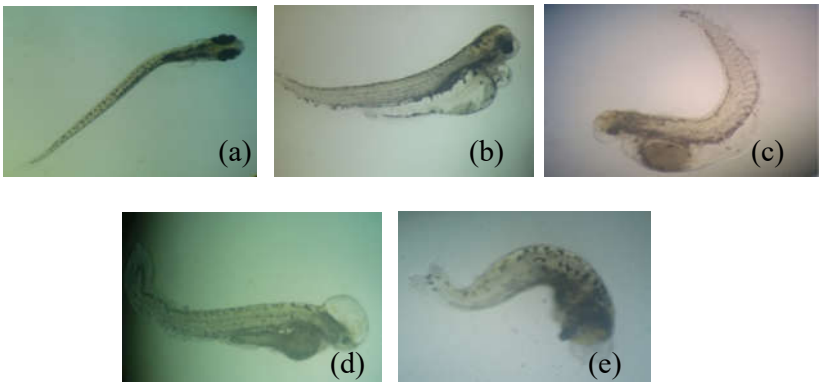


Figure 3: **Observed effects on144hpf larvae irradiated at different doses: (a) 10Gy; (b) 20Gy; (c) 30Gy; (d) 40Gy and (e) 50Gy.**

In the Fig. 3 is presented effect types observed after gamma irradiation at different doses. Geiger [13] and McAleer [14] demonstrated that the abnormalities effects caused by the exposition to gamma radiation are curvature of the spine, shortening of the overall length of the body, pericardial edema, inhibition of yolk sac resorption, micro-ophthalmia, and microcephaly, as observed in this assay.

The effects on morphology and abnormalities increase as the irradiation doses increase and the age of the embryo is an important factor at the time of irradiation [13].

3.2 Cytotoxicity assay

The results of resveratrol cytotoxicity are presented in the Fig.4. All the viability curves above 50% viability (IC50 line) is considered non cytotoxic, as the negative control and the curves that cross or is underthe IC50 line is considered cytotoxic, like positive control. Theobtained resveratrol IC_{50}in the Fig. 4 graphicwas 28% corresponding to 69µM because resveratrol 100% was 250 µM.Positive control IC50 was 72%.IC50 is the sample concentration which provoke injury or death on 50% of cellular population in the assay.

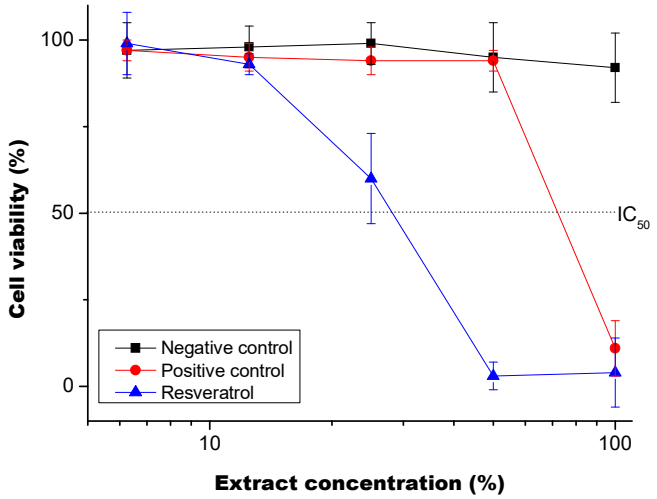


Figure 4 : Viability curves in the resveratrol cytotoxicity assay by NRU method.

3.3 Resveratrol acute toxicity test on*Danio rerio* embryos (LC50)

The resveratrol LC50 on *Danio rerio* embryos was 66.99 $mg.L^{-1}$. LC50 is the concentration of the sample that induce lethality to 50% of exposedorganismsin the assay. . The reported lethal endpoint was the coagulation of the eggs. The used positive control was100 $mg.L^{-1}$ $ZnCl_2$, this solution was toxic to 100% of the organismsexposed in this plate. The DMSO and negative controls didnot provoke any mortality. In theFig. 5 we can see that in the lower concentration (6.25 $mg.L^{-1}$)there was no mortality and in the highest (100 $mg.L^{-1}$) the mortality was 100%.

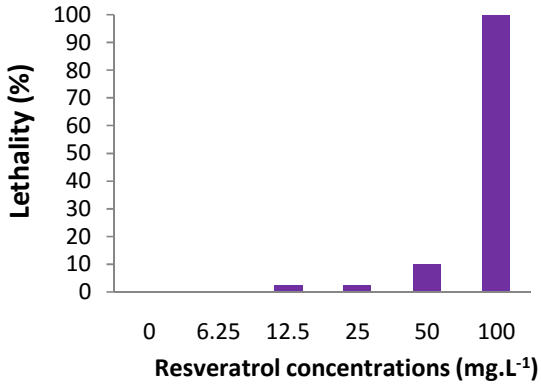


Figure 5: Resveratrol acute toxicity test on*Danio rerio* embryos.Results oflethality in function of resveratrolconcentrations.

The Fig.6 show the effect of resveratrol in toxic concentration on zebrafish embryos.

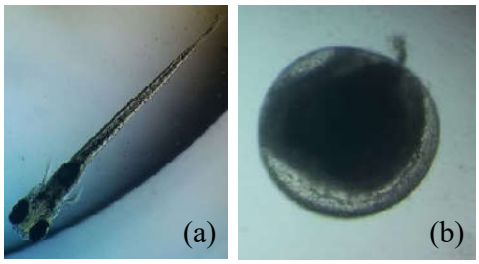


Figure 6: Danio rerio larvae after 96 h of explosion in the acute toxicity assay. (a) Control: exposition to dillution medium (b) Coagulated embryo: exposition to toxic resveratrol solution (100 $mg.L^{-1}$).

4. CONCLUSIONS

The zebrafish is an important model to study the effects of radiomodifier substances, in the *in vivo* test.Although there are some data about how ionizing radiation affects the embyos, it is important to know the toxicity level of radiation and the radiomodifier compound. The data reported on this work are the base for making adjustments to continue the study and provide more results about the interaction of resveratrol with a living organisms when exposed to gamma radiation and provide more information of aradiomodifier agent that could be used in the therapeutic or in the accidental radiation exposure.

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REFERENCES

1. Frémont, Lucie. "Biological effects of resveratrol". *Life sciences*, **v. 66**, n. 8, p. 663-673, 2000.
2. Anisimova et al. "Trans-, cis-, and dihydro-resveratrol: a comparative study Chemistry". *Central Journal* 2011
3. Emília juan M. et al."Trans-Resveratrol, a Natural Antioxidant from Grapes, Increases Sperm Output in Healthy Rats.*The Journal of Nutrition: American Society for Nutrition"* Apr 1, 2005.
4. Carsten, R.E., Bachand, A.M., Bailey, S.M., Ullrich, R.L. "Resveratrol Reduces Radiation-Induced Chromosome Aberration Frequencies in Mouse Bone Marrow Cells. *Radiation research*". 2008
5. Moreno, C.S. "Estudo do efeito radioprotetor do resveratrol". 2009. Dissertação (Mestrado em Tecnologia Nuclear - Aplicações) - Instituto de Pesquisas Energéticas e Nucleares, Universidade de São Paulo, São Paulo, 2009.
6. Magalhães, V.D. "Efeito radiomodificador do resveratrol em cultura de células de rabdomiossarcoma humano (RD) aplicando o teste do cometa". 2012. Dissertação (Mestrado em Tecnologia Nuclear - Aplicações) - Instituto de Pesquisas Energéticas e Nucleares, Universidade de São Paulo, São Paulo, 2012.
7. Barrionuevo, W.R."Zebrafish, Danio rerio, reared under normoxic and hypoxic conditions and exposed to acute hypoxia during development"**.** 2010. *Brazilian Journal of Biology*, **v.70**, n.2, p.425-434, 2010.
8. Lu, Bo et al. "Zebrafish as a model system to screen radiation modifiers". Current genomics, **v. 8**, n. 6, p. 360-369, 2007.
9. OECD (2013), *Test No. 236: Fish Embryo Acute Toxicity (FET) Test*, OECD Publishing, Paris.

10. Hamilton, Martin A., Rosemarie C. Russo, and Robert V. Thurston. "Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays." *Environmental Science & Technology* 11.7 (1977): 714-719.
11. INTERNATION ORGANIZATION FOR STANDARDIZATION.Biological evaluation of medical devices, part5, tests for cytotoxicity: in vitromethods**.(**ISO 10993-5).1999
12. Daroczi, Borbala et al. “In vivo radioprotection by the fullerene nanoparticle DF-1 as assessed in a zebrafish model”. Clinical Cancer Research, **v. 12**, n. 23, p. 7086-7091, 2006.
13. Geiger, Geoffrey A. et al. “Zebrafish as a biosensor ?Effects of ionizing radiation and amifostine on embryonic viability and development”. Cancer research, **v. 66**, n. 16, p. 8172-8181, 2006.
14. 14. Mcaleer, Mary Frances et al. “Novel use of zebrafish as a vertebrate model to screen radiation protectors and sensitizers”. International Journal of Radiation Oncology* Biology* Physics, **v. 61**, n. 1, p. 10-13, 2005.